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(54) Title: MODIFIED FACTOR VII

(57) Abstract

The catalytic active site of Factor VII is modified to produce a compound which effectively interrupts the blood coagulation cascade. The modification render Factor VIIa substantially unable to activate plasma Factors X or IX. Pharmaceutical compositions of the modified Factor VII are used to treat a variety of coagulation-related disorders.

MODIFIED FACTOR VII

Related Applications

The present invention is a continuation-in-part of USSN filed May 21, 1993, which is incorporated herein by reference.

Field of the Invention

The present invention relates to proteins useful as anticoagulants. More specifically, the present invention relates to modified forms of Factor VII that inhibit blood coagulation and tissue factor.

Background Of The Invention

Blood coagulation is a process consisting of a complex interaction of various blood components, or factors, which eventually gives rise to a fibrin clot. Generally, the blood components which participate in what has been referred to as the coagulation "cascade" are proenzymes or zymogens, enzymatically inactive proteins which are converted to proteolytic enzymes by the action of an activator, itself an activated clotting factor. Coagulation factors that have undergone such a conversion and generally referred to as "active factors," and are designated by the addition of a lower case "a" suffix (e.g., Factor VIIa).

Activated Factor X ("Xa") is required to convert prothrombin to thrombin, which then converts fibrinogen to fibrin as a final stage in forming a fibrin clot. There are two systems, or pathways, that promote the activation of Factor X. The "intrinsic pathway" refers to those reactions that lead to thrombin formation through utilization of factors present only in plasma. A series of protease-mediated activations ultimately generates Factor IXa which, in conjunction with Factor VIIIa, cleaves Factor X into Xa. An identical proteolysis is effected by Factor VIIa and its co-factor, tissue factor, in the "extrinsic pathway" of blood

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coagulation. Tissue factor is a membrane bound protein and
does not normally circulate in plasma. Upon vessel
disruption, however, it can complex with Factor VIIa to
catalyze Factor X activation or Factor IX activation in the
5   presence of Ca^{++} and phospholipid (Nemerson and Gentry,
Biochem. 25:4020-4033 (1986)). While the relative importance
of the two coagulation pathways in hemostasis is unclear, in
recent years Factor VII and tissue factor have been found to
play a pivotal role in the regulation of blood coagulation.
10   Factor VII is a trace plasma glycoprotein that
circulates in blood as a single-chain zymogen. The zymogen is
catalytically inactive (Williams et al., J. Biol. Chem.
264:7536-7543 (1989); Rao et al., Proc. Natl. Acad. Sci. USA.
85:6687-6691 (1988)). Single-chain Factor VII may be
15   converted to two-chain Factor VIIa by Factor Xa, Factor XIIa,
Factor IXa or thrombin in vitro. Factor Xa is believed to be
the major physiological activator of Factor VII. Like several
other plasma proteins involved in hemostasis, Factor VII is
dependent on vitamin K for its activity, which is required for
20   the γ-carboxylation of multiple glutamic acid residues that
are clustered in the amino terminus of the protein. These γ-
carboxylated glutamic acids are required for the metal-
associated interaction of Factor VII with phospholipids.
The conversion of zymogen Factor VII into the
25   activated two-chain molecule occurs by cleavage of an internal
peptide bond located approximately in the middle of the
molecule. In human Factor VII, the activation cleavage site
is at Arg_{152}-Ile_{153} (Hagen et al., Proc. Natl. Acad. Sci. USA
83: 2412-2416 (1986); Thim et al., Biochem. 27:7785-7793
30   (1988) both of which are incorporated herein by references).
Bovine factor VII is activated by cleavage at the analogous
Arg_{152}-Ile_{153} bond (Takeya et al., J. Biol. Chem. 263: 14868-
14877, 1988). In the presence of tissue factor, phospholipids
and calcium ions, the two-chain Factor VIIa rapidly activates
35   Factor X or Factor IX by limited proteolysis.
It is often necessary to selectively block the
coagulation cascade in a patient. Anticoagulants such as

heparin, coumarin, derivatives of coumarin, indandione
derivatives, or other agents may be used, for example, during
kidney dialysis, or to treat deep vein thrombosis,
disseminated intravascular coagulation (DIC), and a host of
other medical disorders. For example, heparin treatment or
extracorporeal treatment with citrate ion (U.S. Patent
4,500,309) may be used in dialysis to prevent coagulation
during the course of treatment. Heparin is also used in
preventing deep vein thrombosis in patients undergoing
surgery.

Treatment with heparin and other anticoagulants
may, however, have undesirable side effects. Available
anticoagulants generally act throughout the body, rather than
acting specifically at a clot site. Heparin, for example, may
cause heavy bleeding. Furthermore, with a half-life of
approximately 80 minutes, heparin is rapidly cleared from the
blood, necessitating frequent administration. Because heparin
acts as a cofactor for antithrombin III (AT III), and AT III
is rapidly depleted in DIC treatment, it is often difficult to
maintain the proper heparin dosage, necessitating continuous
monitoring of AT III and heparin levels. Heparin is also
ineffective if AT III depletion is extreme. Further,
prolonged use of heparin may also increase platelet
aggregation and reduce platelet count, and has been implicated
in the development of osteoporosis. Indandione derivatives
may also have toxic side effects.

In addition to the anticoagulants briefly described
above, several naturally occurring proteins have been found to
have anticoagulant activity. For example, Reutelingsperger
(U.S. Patent No. 4,736,018) isolated anticoagulant proteins
from bovine aorta and human umbilical vein arteries. Maki et
al. (U.S.Patent No. 4,732,891) disclose human placenta-derived
anticoagulant proteins. In addition, AT III has been proposed
as a therapeutic anticoagulant (Schipper et al., Lancet 1
(8069): 854-856 (1978); Jordan, U.S. Patent No. 4,386,025;
Bock et al., U.S. Patent No. 4,517,294).

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Proliferation of smooth muscle cells (SMCs) in the vessel wall is an important event in the formation of vascular lesions in atherosclerosis, after vascular reconstruction or in response to other vascular injury. For example, treatment of atherosclerosis frequently includes the clearing of blocked vessels by angioplasty, endarterectomy or reduction atherectomy, or by bypass grafting, surgical procedures in which atherosclerotic plaques are compressed or removed through catheterization (angioplasty), stripped away from the arterial wall through an incision (endarterectomy) or bypassed with natural or synthetic grafts. These procedures remove the vascular endothelium, disturb the underlying intimal layer, and result in the death of medial SMCs. This injury is followed by medial SMC proliferation and migration into the intima, which characteristically occurs within the first few weeks and up to six months after injury and stops when the overlying endothelial layer is reestablished. In humans, these lesions are composed of about 20% cells and 80% extracellular matrix.

In about 30% or more of patients treated by angioplasty, endarterectomy or bypass grafts, thrombosis and/or SMC proliferation in the intima causes re-occlusion of the vessel and consequent failure of the reconstructive surgery. This closure of the vessel subsequent to surgery is known as restenosis.

There is still a need in the art for improved compositions having anticoagulant activity which can be administered at relatively low doses and do not produce the undesirable side effects associated with traditional anticoagulant compositions. The present invention fulfills this need by providing anticoagulants that act specifically at sites of injury, and further provides other related advantages.

Summary Of The Invention

Novel compositions which comprise modified Factor VII having anticoagulant properties are provided. The modified Factor VII compositions also inhibit tissue factor. The Factor VII sequence has at least one amino acid modification, where the modification is selected so as to substantially reduce the ability of activated Factor VII to catalyze the activation of plasma Factors X or IX, and thus is capable of inhibiting clotting activity. The novel Factor VII has an active site modified by at least one amino acid substitution, and in its modified form is capable of binding tissue factor. The modified Factor VII compositions are typically in substantially pure form.

The compositions of the invention are particularly useful in methods for treating patients when formulated into pharmaceutical compositions, where they may be given to individuals suffering from a variety of disease states to treat coagulation-related conditions. Such modified Factor VII molecules, capable of binding tissue factor but having a substantially reduced ability to catalyze activation of other factors in the clotting cascade, may possess a longer plasma half-life and thus a correspondingly longer period of anticoagulative activity when compared to other anticoagulants. Among the medical indications for the subject compositions are those commonly treated with anticoagulants, such as, for example, deep vein thrombosis, pulmonary embolism, stroke, disseminated intravascular coagulation (DIC) and myocardial infarction. The compositions can be used to inhibit vascular restenosis and platelet deposition and associated disorders. Thus, a method of inhibiting coagulation, vascular restenosis or platelet deposition, for example, comprises administering to a patient a composition comprising modified Factor VII, such as that having at least one amino acid substitution in a catalytic triad of Ser_{344}, Asp_{242} and His_{193}, in an amount sufficient to effectively inhibit coagulation, vascular restenosis or platelet deposition, respectively.

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Typically, for administration to humans the
pharmaceutical compositions will comprise modified human
Factor VII protein and pharmaceutically-acceptable carriers
and buffers.

In preferred embodiments of human and bovine Factor
VII, the active site residue Ser_{344} is modified, replaced with
Gly, Met, Thr, or more preferably, Ala. Such substitution
could be made separately or in combination with
substitution(s) at other sites in the catalytic triad, which
includes His_{193} and Asp_{242}.

In another aspect the invention relates to a
polynucleotide molecule comprising two operatively linked
sequence coding regions encoding, respectively, a pre-pro
peptide and a gla domain of a vitamin K-dependent plasma
protein, and a gla domain-less Factor VII protein, wherein
upon expression said polynucleotide encodes a modified Factor
VII molecule which does not significantly activate plasma
Factors X or IX, and is capable of binding tissue factor. The
modified Factor VII molecule expressed by this polynucleotide
is a biologically active anticoagulant, that is, it is capable
of inhibiting the coagulation cascade and thus the formation
of a fibrin deposit or clot. To express the modified Factor
VII the polynucleotide molecule is transfected into mammalian
cell lines, such as, for example, BHK, BHK 570 or 293 cell
lines.

Brief Description Of The Figure

The Figure illustrates the construction of an
expression vector for a $Ser_{344}{\rightarrow}Ala$ modified Factor VII DNA
sequence. Symbols used include 0-1, the 0-1 map unit sequence
from adenovirus 5; E, the SV40 enhancer; MLP, the adenovirus 2
major late promotor; SS, a set of splice sites; and pA, the
polyadenylation signal from SV40 in the late orientation.

Description Of The Specific Embodiments

Novel modified Factor VII having anticoagulant
activity is provided by the present invention. The modified

Factor VII can be in the form of the zymogen (i.e., a single-
chain molecule) or can be cleaved at its activation site.
Compositions of the modified Factor VII are suitable for
administration to a variety of mammals, particularly humans,
to inhibit the coagulation cascade. Modified Factor VII may
be administered to a patient in conjunction with or in place
of other anticoagulant compounds.

          Factor VII plays an important role in the
coagulation cascade, particularly that involving the extrinsic
pathway. Present in the circulating plasma as an inactive
single chain zymogen protein, once activated, Factor VIIa, in
combination with tissue factor and calcium ions, activates
Factor X to Xa and activates Factor IX to IXa, with the
eventual formation of a fibrin clot.

          The present invention provides the ability to
inhibit this sequence of events in the coagulation cascade by
preventing or otherwise inhibiting the activation of Factors X
and IX by Factor VIIa. Factor VII proteins of the invention
have a catalytic site which is modified to decrease the
catalytic activity of Factor VIIa, while the molecule retains
the ability to bind to tissue factor. The modified Factor VII
molecules compete with native Factor VII and/or VIIa for
binding to tissue factor. As a result, the activation of
Factors X and IX is inhibited.

          In one aspect of the present invention the catalytic
activity of Factor VIIa is inhibited by chemical
derivatization of the catalytic center, or triad.
Derivatization may be accomplished by reacting Factor VII with
an irreversible inhibitor such as an organophosphor compound,
a sulfonyl fluoride, a peptide halomethyl ketone or an
azapeptide, or by acylation, for example. Preferred peptide
halomethyl ketones include PPACK (D-Phe-Pro-Arg chloromethyl
ketone; see U.S. Patent No. 4,318,904, incorporated herein by
reference), and DEGRck (dansyl-Glu-Gly-Arg chloromethyl
ketone).

          In another aspect, the catalytic activity of Factor
VIIa can also be inhibited by substituting, inserting or

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deleting amino acids. In preferred embodiments amino acid
substitutions are made in the amino acid sequence of the
Factor VII catalytic triad, defined herein as the regions
which contain the amino acids which contribute to the Factor

5   VIIa catalytic site. The substitutions, insertions or
deletions in the catalytic triad are generally at or adjacent
to the amino acids which form the catalytic site. In the
human and bovine Factor VII proteins, the amino acids which
form a catalytic "triad" are Ser_{344}, Asp_{242}, and His_{193}

10   (subscript numbering indicating position in the sequence).
The catalytic sites in Factor VII from other mammalian species
may be determined using presently available techniques
including, among others, protein isolation and amino acid
sequence analysis. Catalytic sites may also be determined by

15   aligning a sequence with the sequence of other serine
proteases, particularly chymotrypsin, whose active site has
been previously determined (Sigler et al., J. Mol. Biol.,
35:143-164 (1968), incorporated herein by reference), and
therefrom determining from said alignment the analogous active

20   site residues.
          The amino acid substitutions, insertions or
deletions are made so as to prevent or otherwise inhibit
activation by the Factor VIIa of Factors X and/or IX. The
Factor VII so modified should, however, also retain the

25   ability to compete with authentic Factor VII and/or Factor
VIIa for binding to tissue factor in the coagulation cascade.
Such competition may readily be determined by means of, e.g.,
a clotting assay as described herein, or a competition binding
assay using, e.g., a cell line having cell-surface tissue

30   factor, such as the human bladder carcinoma cell line J82
(Sakai et al. J. Biol. Chem. 264: 9980-9988 (1989),
incorporated by reference herein.)
          The amino acids which form the catalytic site in
Factor VII, such as Ser_{344}, Asp_{242}, and His_{193} in human and

35   bovine Factor VII, may either be substituted or deleted.
Within the present invention, it is preferred to change only a
single amino acid, thus minimizing the likelihood of

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increasing the antigenicity of the molecule or inhibiting its
ability to bind tissue factor, however two or more amino acid
changes (substitutions, additions or deletions) may be made
and combinations of substitution(s), addition(s) and

5   deletion(s) may also be made. In a preferred embodiment for
human and bovine Factor VII, Ser_{344} is preferably substituted
with Ala, but Gly, Met, Thr or other amino acids can be
substituted. It is preferred to replace Asp with Glu and to
replace His with Lys or Arg. In general, substitutions are

10   chosen to disrupt the tertiary protein structure as little as
possible. The model of Dayhoff et al. (in <u>Atlas of Protein
Structure 1978</u>, Nat'l Biomed. Res. Found., Washington, D.C.),
incorporated herein by reference, may be used as a guide in
selecting other amino acid substitutions. One may introduce

15   residue alterations as described above in the catalytic site
of appropriate Factor VII sequence of human, bovine or other
species and test the resulting protein for a desired level of
inhibition of catalytic activity and resulting anticoagulant
activity as described herein. For the modified Factor VII the

20   catalytic activity will be substantially inhibited, generally
less than about 5% of the catalytic activity of wild-type
Factor VII of the corresponding species, more preferably less
than about 1%.

       The proteins of the present invention may be

25   produced through the use of recombinant DNA techniques. In
general, a cloned wild-type Factor VII DNA sequence is
modified to encode the desired protein. This modified
sequence is then inserted into an expression vector, which is
in turn transformed or transfected into host cells. Higher

30   eukaryotic cells, in particular cultured mammalian cells, are
preferred as host cells. The complete nucleotide and amino
acid sequences for human Factor VII are known. See U.S. Pat.
No. 4,784,950, which is incorporated herein by reference,
where the cloning and expression of recombinant human Factor

35   VII is described. The bovine Factor VII sequence is described
in Takeya et al., <u>J. Biol. Chem.</u> 263:14868-14872 (1988), which
is incorporated by reference herein.

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The amino acid sequence alterations may be
accomplished by a variety of techniques. Modification of the
DNA sequence may be by site-specific mutagenesis. Techniques
for site-specific mutagenesis are well known in the art and
5   are described by, for example, Zoller and Smith (DNA 3:479-
488, 1984). Thus, using the nucleotide and amino acid
sequences of Factor VII, one may introduce the alteration(s)
of choice.

The Factor VII modified according to the present
10   invention includes those proteins that have the amino-terminal
portion (gla domain) substituted with a gla domain of one of
the vitamin K-dependent plasma proteins Factor IX, Factor X,
prothrombin, protein C, protein S or protein Z. The gla
domains of the vitamin K-dependent plasma proteins are
15   characterized by the presence of gamma-carboxy glutamic acid
residues and are generally from about 30 to about 40 amino
acids in length with C-termini corresponding to the positions
of exon-intron boundaries in the respective genes. Methods
for producing Factor VII with a heterologous gla domain are
20   disclosed in U.S. Patent No. 4,784,950, incorporated by
reference herein.

DNA sequences for use within the present invention
will typically encode a pre-pro peptide at the amino-terminus
of the Factor VII protein to obtain proper post-translational
25   processing (e.g. gamma-carboxylation of glutamic acid
residues) and secretion from the host cell. The pre-pro
peptide may be that of Factor VII or another vitamin K-
dependent plasma protein, such as Factor IX, Factor X,
prothrombin, protein C or protein S. As will be appreciated
30   by those skilled in the art, additional modifications can be
made in the amino acid sequence of the modified Factor VII
where those modifications do not significantly impair the
ability of the protein to act as an anticoagulant. For
example, the Factor VII modified in the catalytic triad can
35   also be modified in the activation cleavage site to inhibit
the conversion of zymogen Factor VII into its activated two-

chain form, as generally described in U.S. Patent 5,288,629,
incorporated herein by reference.

Expression vectors for use in carrying out the
present invention will comprise a promoter capable of
directing the transcription of a cloned gene or cDNA.
Preferred promoters for use in cultured mammalian cells
include viral promoters and cellular promoters. Viral
promoters include the SV40 promoter (Subramani et al., Mol.
Cell. Biol. 1:854-864, 1981) and the CMV promoter (Boshart et
al., Cell 41:521-530, 1985). A particularly preferred viral
promoter is the major late promoter from adenovirus 2 (Kaufman
and Sharp, Mol. Cell. Biol. 2:1304-1319, 1982). Cellular
promoters include the mouse kappa gene promoter (Bergman et
al., Proc. Natl. Acad. Sci. USA 81:7041-7045, 1983) and the
mouse V_H promoter (Loh et al., Cell 33:85-93, 1983). A
particularly preferred cellular promoter is the mouse
metallothionein-I promoter (Palmiter et al., Science 222:809-
814, 1983). Expression vectors may also contain a set of RNA
splice sites located downstream from the promoter and upstream
from the insertion site for the Factor VII sequence itself.
Preferred RNA splice sites may be obtained from adenovirus
and/or immunoglobulin genes. Also contained in the expression
vectors is a polyadenylation signal located downstream of the
insertion site. Particularly preferred polyadenylation
signals include the early or late polyadenylation signal from
SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal
from the adenovirus 5 Elb region, the human growth hormone
gene terminator (DeNoto et al. Nuc. Acids Res. 9:3719-3730,
1981) or the polyadenylation signal from the human Factor VII
gene or the bovine Factor VII gene. The expression vectors
may also include a noncoding viral leader sequence, such as
the adenovirus 2 tripartite leader, located between the
promoter and the RNA splice sites; and enhancer sequences,
such as the SV40 enhancer.

Cloned DNA sequences are introduced into cultured
mammalian cells by, for example, calcium phosphate-mediated
transfection (Wigler et al., Cell 14:725-732, 1978; Corsaro

and Pearson, Somatic Cell Genetics 7:603-616, 1981; Graham and
Van der Eb, Virology 52d:456-467, 1973) or electroporation
(Neumann et al., EMBO J. 1:841-845, 1982). To identify and
select cells that express the exogenous DNA, a gene that
confers a selectable phenotype (a selectable marker) is
generally introduced into cells along with the gene or cDNA of
interest. Preferred selectable markers include genes that
confer resistance to drugs such as neomycin, hygromycin, and
methotrexate. The selectable marker may be an amplifiable
selectable marker. A preferred amplifiable selectable marker
is a dihydrofolate reductase (DHFR) sequence. Selectable
markers are reviewed by Thilly (Mammalian Cell Technology,
Butterworth Publishers, Stoneham, MA, incorporated herein by
reference). The choice of selectable markers is well within
the level of ordinary skill in the art.

Selectable markers may be introduced into the cell
on a separate plasmid at the same time as the gene of
interest, or they may be introduced on the same plasmid. If
on the same plasmid, the selectable marker and the gene of
interest may be under the control of different promoters or
the same promoter, the latter arrangement producing a
dicistronic message. Constructs of this type are known in the
art (for example, Levinson and Simonsen, U.S. Patent
4,713,339). It may also be advantageous to add additional
DNA, known as "carrier DNA," to the mixture that is introduced
into the cells.

After the cells have taken up the DNA, they are
grown in an appropriate growth medium, typically 1-2 days, to
begin expressing the gene of interest. As used herein the
term "appropriate growth medium" means a medium containing
nutrients and other components required for the growth of
cells and the expression of the modified Factor VII gene.
Media generally include a carbon source, a nitrogen source,
essential amino acids, essential sugars, vitamins, salts,
phospholipids, protein and growth factors. For production of
gamma-carboxylated modified Factor VII, the medium will
contain vitamin K, preferably at a concentration of about 0.1

μg/ml to about 5 μg/ml. Drug selection is then applied to
select for the growth of cells that are expressing the
selectable marker in a stable fashion. For cells that have
been transfected with an amplifiable selectable marker the

5   drug concentration may be increased to select for an increased
copy number of the cloned sequences, thereby increasing
expression levels. Clones of stably transfected cells are
then screened for expression of modified Factor VII.

Preferred mammalian cell lines for use in the

10   present invention include the COS-1 (ATCC CRL 1650), baby
hamster kidney (BHK) and 293 (ATCC CRL 1573; Graham et al., _J.
Gen. Virol_. 36:59-72, 1977) cell lines. A preferred BHK cell
line is the tk⁻ ts13 BHK cell line (Waechter and Baserga,
Proc. Natl. Acad. Sci. USA 79:1106-1110, 1982, incorporated

15   herein by reference), hereinafter referred to as BHK 570
cells. The BHK 570 cell line has been deposited with the
American Type Culture Collection, 12301 Parklawn Dr.,
Rockville, MD 20852, under ATCC accession number CRL 10314.
A tk⁻ ts13 BHK cell line is also available from the ATCC under

20   accession number CRL 1632. In addition, a number of other
cell lines may be used within the present invention, including
Rat Hep I (Rat hepatoma; ATCC CRL 1600), Rat Hep II (Rat
hepatoma; ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung
(ATCC HB 8065), NCTC 1469 (ATCC CCL 9.1), CHO (ATCC CCL 61)

25   and DUKX cells (Urlaub and Chasin, _Proc. Natl. Acad. Sci. USA_
77:4216-4220, 1980).

Modified Factor VII produced according to the
present invention may be purified by affinity chromatography
on an anti-Factor VII antibody column. The use of calcium-

30   dependent monoclonal antibodies, as described by Wakabayashi
et al., _J. Biol. Chem._ 261:11097-11108, (1986) and Thim et
al., _Biochem._ 27: 7785-7793, (1988), incorporated by reference
herein, is particularly preferred. Additional purification
may be achieved by conventional chemical purification means,

35   such as high performance liquid chromatography. Other methods
of purification, including barium citrate precipitation, are
known in the art, and may be applied to the purification of

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the novel modified Factor VII described herein (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y., 1982). Substantially pure modified Factor VII of at least about 90 to 95% homogeneity is preferred, and 98 to 99%

5   or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the modified Factor VII may then be used therapeutically.

Within one embodiment of the invention the modified Factor VII is cleaved at its activation site to convert it to

10   its two-chain form. Activation may be carried out according to procedures known in the art, such as those disclosed by Osterud, et al., Biochemistry 11:2853-2857 (1972); Thomas, U.S. Patent No. 4,456,591; Hedner and Kisiel, J. Clin. Invest. 71:1836-1841 (1983); or Kisiel and Fujikawa, Behring Inst.

15   Mitt. 73:29-42 (1983), which are incorporated herein by reference. The resulting modified "Factor VIIa" is then formulated and administered as described below.

The modified Factor VII or VIIa molecules of the present invention and pharmaceutical compositions thereof are

20   particularly useful for administration to humans to treat a variety of conditions involving intravascular coagulation. For example, although deep vein thrombosis and pulmonary embolism can be treated with conventional anticoagulants, the modified Factor VII or VIIa described herein may be used to

25   prevent the occurrence of thromboembolic complications in identified high risk patients, such as those undergoing surgery or those with congestive heart failure. In addition, modified Factor VII or VIIa may act as an antagonist for tissue factor-mediated induction of coagulation, thus blocking

30   the production of thrombin and the subsequent deposition of fibrin. As such, modified Factor VII or VIIa may be useful for inhibiting tissue factor activity resulting in, for example, the inhibition of blood coagulation, thrombosis or platelet deposition.

35   The modified Factor VII or VIIa molecules of the present invention may be particularly useful in the treatment of intimal hyperplasia or restenosis due to acute vascular

injury. Acute vascular injuries are those which occur rapidly
(i.e. over days to months), in contrast to chronic vascular
injuries (e.g. atherosclerosis) which develop over a lifetime.
Acute vascular injuries often result from surgical procedures
such as vascular reconstruction, wherein the techniques of
angioplasty, endarterectomy, atherectomy, vascular graft
emplacement or the like are employed. Hyperplasia may also
occur as a delayed response in response to, e.g., graft
emplacement or organ transplantation. Since modified Factor
VII is more selective than heparin, generally binding only
tissue factor which has been exposed at sites of injury, and
because modified Factor VII does not destroy other coagulation
proteins, it will be more effective and less likely to cause
bleeding complications that heparin when used prophylactically
for the prevention of deep vein thrombosis. The dose of
modified Factor VII for prevention of deep vein thrombosis is
in the range of about 50 μg to 25 mg/day, preferably 1 to 10
mg/day for a 70 kg patient, and administration should begin at
least about 6 hours prior to surgery and continue at least
until the patient becomes ambulatory. The dose of modified
Factor VII or VIIa in the treatment for restenosis will vary
with each patient but will generally be in the range of those
suggested above.

Recent advances in the treatment of coronary
vascular disease include the use of mechanical interventions
to either remove or displace offending plaque material in
order to re-establish adequate blood flow through the coronary
arteries. Despite the use of multiple forms of mechanical
interventions, including balloon angioplasty, reduction
atherectomy, placement of vascular stents, laser therapy, or
rotoblator, the effectiveness of these techniques remains
limited by an approximately 40% restenosis rate within 6
months after treatment.

Restenosis is thought to result from a complex
interaction of biological processes including platelet
deposition and thrombus formation, release of chemotactic and
mitogenic factors, and the migration and proliferation of

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vascular smooth muscle cells into the intima of the dilated
arterial segment.

The inhibition of platelet accumulation at sites of
mechanical injury can limit the rate of restenosis in human
subjects.   Therapeutic use of a monoclonal antibody to
platelet GpIIb/IIIa is able to limit the level of restenosis
in human subjects (Califf et al., N. Engl. J. Med., 330:956-
961 (1994)). The antibody is able to bind to the GpIIb/IIIa
receptor on the surfaces of platelets and thereby inhibit
platelet accumulation. This data suggests that inhibition of
platelet accumulation at the site of mechanical injury in
human coronary arteries is beneficial for the ultimate healing
response that occurs. While platelet accumulation occurs at
sites of acute vascular injuries, the generation of thrombin
at these sites may be responsible for the activation of the
platelets and their subsequent accumulation.

As shown in the examples that follow, the modified
Factor VIIa of the present invention is able to bind to cell-
surface tissue factor. For example, DEGR-Factor VIIa binds
cell-surface tissue factor with an equivalent or higher
affinity than wild-type Factor VIIa. DEGR-Factor VIIa,
however, has no enzymatic activity, yet it binds to tissue
factor and acts as a competitive antagonist for wild-type
Factor VIIa, thereby inhibiting the subsequent steps in the
extrinsic pathway of coagulation leading to the generation of
thrombin.

Modified Factor VIIa molecules of the present
invention which maintain tissue factor inhibit platelet
accumulation at the site of vascular injury by blocking the
production of thrombin and the subsequent deposition of
fibrin.

Due to the ability of DEGR-Factor VIIa to block
thrombin generation and limit platelet deposition at sites of
acute vascular injury, modified Factor VIIa molecules maintain
tissue factor binding activity but lack Factor VIIa enzymatic
activity can be used to inhibit vascular restenosis.

In established deep vein thrombosis and/or pulmonary
embolism, the dose of modified Factor VII ranges from about 50
μg to 25 mg as a loading dose followed by maintenance doses
ranging from about 500 μg to 10 mg/day, depending on the
5   weight of the patient and the severity of the condition.
Because of the lower likelihood of bleeding complications from
modified Factor VII infusions, modified Factor VII can replace
or lower the dose of heparin during or after surgery in
conjunction with thrombectomies or embolectomies.
10   The modified Factor VII compositions of the present
invention will also have substantial utility in the prevention
of cardiogenic emboli and in the treatment of thrombotic
strokes. Because of its low potential for causing bleeding
complications and its selectivity, modified Factor VII can be
15   given to stroke victims and may prevent the extension of the
occluding arterial thrombus. The amount of modified Factor
VII administered will vary with each patient depending on the
nature and severity of the stroke, but doses will generally be
in the range of those suggested below.
20   Pharmaceutical compositions of modified Factor VII
provided herein will be a useful treatment in acute myocardial
infarction because of the ability of modified Factor VII to
inhibit in vivo coagulation. Modified Factor VII can be given
with tissue plasminogen activator or streptokinase during the
25   acute phases of the myocardial infarction. In acute
myocardial infarction, the patient is given a loading dose of
at least about 1 to 25 mg of modified Factor VII, followed by
maintenance doses of about 500 μg to about 10 mg/day.
               The modified Factor VII of the present invention is
30   useful in the treatment of disseminated intravascular
coagulation (DIC). Patients with DIC characteristically have
widespread microcirculatory thrombi and often severe bleeding
problems which result from consumption of essential clotting
factors. Because of its selectivity, modified Factor VII will
35   not aggravate the bleeding problems associated with DIC, as do
conventional anticoagulants, but will retard or inhibit the
formation of additional microvascular fibrin deposits.

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        The pharmaceutical compositions are intended for
parenteral, topical or local administration for prophylactic
and/or therapeutic treatment. Preferably, the pharmaceutical
compositions are administered parenterally, i.e.,
5   intravenously, subcutaneously, or intramuscularly. Thus, this
invention provides compositions for parenteral administration
which comprise a solution of the modified Factor VII molecules
dissolved in an acceptable carrier, preferably an aqueous
carrier. A variety of aqueous carriers may be used, e.g.,
10   water, buffered water, 0.4% saline, 0.3% glycine and the like.
The modified Factor VII molecules can also be formulated into
liposome preparations for delivery or targeting to sites of
injury. Liposome preparations are generally described in,
e.g., U.S. 4,837,028, U.S. 4,501,728, and U.S. 4,975,282,
15   incorporated herein by reference. The compositions may be
sterilized by conventional, well known sterilization
techniques. The resulting aqueous solutions may be packaged
for use or filtered under aseptic conditions and lyophilized,
the lyophilized preparation being combined with a sterile
20   aqueous solution prior to administration. The compositions
may contain pharmaceutically acceptable auxiliary substances
as required to approximate physiological conditions, such as
pH adjusting and buffering agents, tonicity adjusting agents
and the like, for example, sodium acetate, sodium lactate,
25   sodium chloride, potassium chloride, calcium chloride, etc.
The concentration of modified Factor VII in these formulations
can vary widely, i.e., from less than about 0.5%, usually at
or at least about 1% to as much as 15 or 20% by weight and
will be selected primarily by fluid volumes, viscosities,
30   etc., in accordance with the particular mode of administration
selected.
        Thus, a typical pharmaceutical composition for
intravenous infusion could be made up to contain 250 ml of
sterile Ringer's solution, and 10 mg of modified Factor VII.
35   Actual methods for preparing parenterally administrable
compounds will be known or apparent to those skilled in the
art and are described in more detail in for example,

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<u>Remington's Pharmaceutical Science</u>, 16th ed., Mack Publishing Company, Easton, PA (1982), which is incorporated herein by reference.

The compositions containing the modified Factor VII molecules can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease or injury and the weight and general state of the patient, but generally range from about 0.05 mg to about 25 mg of modified Factor VII per day for a 70 kg patient, with dosages of from about 0.5 mg to about 10 mg of modified Factor VII per day being more commonly used. It must be kept in mind that the materials of the present invention may generally be employed in serious disease or injury states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and general lack of immunogenicity of modified human Factor VII in humans, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these modified Factor VII compositions.

In prophylactic applications, compositions containing the modified Factor VII are administered to a patient susceptible to or otherwise at risk of a disease state or injury to enhance the patient's own anticoagulative capabilities. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, but generally range from about 0.1 mg to about 25 mg per 70 kilogram patient, more commonly from about 0.5 mg to about 10 mg per 70 kg of body weight.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern

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being selected by the treating physician. For ambulatory
patients requiring daily maintenance levels, the modified
Factor VII may be administered by continuous infusion using a
portable pump system, for example. Local delivery of the
5   modified Factor VII or VIIa may be carried out, for example,
by way of perfusion, double balloon catheters, stent or other
well established methods. In any event, the pharmaceutical
formulations should provide a quantity of modified Factor VII
of this invention sufficient to effectively treat the patient.
10   The following examples are offered by way of
illustration, not by way of limitation.

EXAMPLE I
Expression of $Ser_{344} \rightarrow Ala_{344}$ Factor VII
15   To generate the $Ser_{344} \rightarrow Ala$ Factor VII active site
mutant, plasmid FVII(565+2463)/ pDX (U.S. Patent No. 4,784,950
incorporated herein by reference; deposited with the American
Type Culture Collection under accession number 40205) was
digested with Xba I and Kpn I, and the resulting 0.6 kb
20   fragment, comprising the coding region for serine 344, was
recovered. This fragment was cloned into Xba I, Kpn I-
digested M13mp19 as shown in the Figure. This manipulation
and subsequent steps described below were generally performed
according to standard protocols (as described, for example, by
25   Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold
Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
(1982) incorporated herein by reference).
            Mutagenesis was carried out on the M13 template
according to the methods of Zoller and Smith, supra, using the
30   mutagenic oligonucleotide ZC1656 (5' TGG GCC TCC GGC GTC CCC
CTT 3') and the "universal" second primer ZC87 (5' TCC CAG TCA
CGA CGT 3'). Reaction products were screened using kinased
ZC1656. Positive plaques were picked, and template DNA was
prepared and sequenced from the Pst I site at 1077 to the Kpn
35   I site at 1213. Sequence analysis confirmed the presence of
the desired mutation. The mutant clone was designated 1656.

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An expression vector was then constructed using the
1656 clone. The mutagenized sequence was isolated from the
M13 vector as a ~0.14 kb Pst I-Kpn I fragment. This fragment
was ligated to the 1.7 kb Hind III-Xba I fragment from
5   FVII(565+2463)/pDX, the 0.5 kb Xba I-Pst I fragment from
FVII(565+2463)/pDX, and the 4.3 kb Kpn I-Hind III fragment
from FVII(565+2463)/pDX, as shown in the Figure. The presence
of the desired mutant sequence was confirmed by digesting
mutant and wild-type clones with Pst I, and a mutant Factor
10   VII insert in M13 with Kpn I and Xba I, preparing Southern
blots of the digested DNA, and probing the blots with
radiolabeled ZC1656.

The baby hamster kidney cell line BHK 570 (deposited
with the American Type Culture Collection under accession
15   number 10314) was transfected with two isolates (designated
#544 and #545) of the 1656 expression vector. The cells were
prepared by diluting a confluent 10 cm plate of BHK 570 cells
1:10 into five 10 cm plates in non-selective medium
(Dulbecco's modified Eagle's medium [DMEM] containing 10%
20   fetal bovine serum and 1% PSN antibiotic mix [GIBCO Life
Technologies, Gaithersburg, MD]). After 24 hours, when the
cells had reached 20-30% confluency, they were co-transfected
with one isolate of the expression vector encoding the 1656
mutation, plasmid p486 (comprising the Adenovirus 5 ori, SV40
25   enhancer, Adenovirus 2 major late promotor, Adenovirus 2
tripartite leader, 5' and 3' splice sites, the DHFR^r cDNA and
SV40 polyadenylation signal in pML-1 (Lusky and Botchan,
Nature 293: 79-81, (1981)) and 10 μg of carrier DNA (sonicated
salmon sperm DNA) as shown in Table 1. The DNA was added to a
30   15 ml tube, then 0.5 ml of 2X Hepes (25 g Hepes, 40 g NaCl,
1.8 g KCl, 0.75 g Na$_2$HPO$_4$·2H$_2$O, 5 g dextrose diluted to 2.5 l
with distilled water and pH adjusted to pH 6.95-7.0) was added
and the tubes were mixed. The DNA in each tube was
precipitated by the addition of 0.5 ml of 0.25 M CaCl$_2$ while
35   air was bubbled through the DNA/Hepes solution with a pasteur
pipet. The tubes were then vortexed; incubated at room
temperature for 15 minutes, and vortexed again. The DNA

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mixtures were then added dropwise onto the plates of cells
with a pipette. The plates were swirled and incubated at 37°C
for 4-6 hours. After incubation, 2 ml of 20% glycerol diluted
in Tris-saline (0.375 g KCl, 0.71 g Na_2HPO_4, 8.1 g NaCl, 3.0 g
Tris-HCl, 0.5 g sucrose, diluted in a total of 1 liter and pH
adjusted to pH 7.9) was then added to each plate. The plates
were swirled and left at room temperature for two minutes.
The medium was then removed from the plates and replaced with
2 ml of Tris-saline. The plates were left at room temperature
for 2 minutes, then the Tris-saline was removed and replaced
with 10 ml of non-selective medium. The plates were incubated
at 37°C for two days.

<div align="center">

Table 1
Transfection*

</div>

Plasmid Name	544	545	544 Control	545 Control
Clone 544	15 μl	---	15 μl	---
Clone 545	---	30 μl	---	30 μl
p486	1.5 μl	1.5 μl	---	---
Carrier DNA	1.6 μl	1.6 μl	1.6 μl	1.6 μl

* DNA concentrations used were: clone 544, 0.7
μg/μl; clone 545, 0.3 μg/μl; p486, 1.49 μg/μl.

After the two day incubation, the cells were diluted
in selection medium (DMEM containing 10% dialyzed fetal bovine
serum, 1% PSN antibiotic mix and 150 nM methotrexate) and
plated at dilutions of 1:100, 1:250 and 1:500 in maxi plates.
The plates were incubated at 37°C for one week. After one
week, the medium was changed and replaced with selection
medium, and the plates were checked for colony formation.
Eight days later, after colony formation, twelve
colonies were randomly chosen from the 1:500 dilution plates
of the #544 and #545 transfection plates. Each clone was
plated into one well of a 6-well plate and grown in selection
medium. After seven days, the plates were confluent, and the
clones were each split into 10 cm plates in selection medium.
The clones described above and control cells
transfected to express wild-type factor VII were metabolically
labeled with ^{35}S-Methionine-Cysteine Protein Labeling Mix (NEN

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DuPont Biotechnology Systems, Wilmington, DE). The clones
were grown and prepared for a pulse label experiment in
selective medium. The cells were rinsed with phosphate
buffered saline (Sigma, St. Louis, MO) and pulsed for four
5   hours in 20 µCi/ml ^{35}S-Cys-^{35}S-Met. After four hours,
supernatants and cells were harvested. The cells were lysed
essentially as described by Lenk and Penman (Cell 16: 289-302,
(1979)) and 400 µl of each lysate and precleared with 50 µl of
staph A (Sigma, St. Louis, MO).
10   Samples from the metabolically labeled cells were
radioimmunoprecipitated (RIP) by first incubating the samples
with 6 µl of anti-Factor VII polyclonal antisera for four
hours. Sixty microliters of washed staphylococcal protein A
was added to each sample, and the samples were rocked for 1.5
15   hours at 4°C. The samples were centrifuged, and the
supernatant was removed. The pellets were washed twice in 0.7
M RIPA buffer (10 mM Tris, pH 7.4, 1% deoxycholic acid
[Calbiochem Corp., La Jolla, CA], 1% Triton X-100, 0.1% SDS, 5
mM EDTA, 0.7 M NaCl) and once in 0.15 M RIPA buffer (10 mM
20   Tris, pH 7.4, 1% deoxycholic acid [Calbiochem Corp., La Jolla,
CA], 1% Triton X-100, 0.1 SDS, 5 mM EDTA, 0.15 M NaCl). One
hundred microliters of 1x SDS dye (50 mM Tris-HCl, pH 6.8, 100
mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol)
was added to each sample, and the samples were boiled for 5
25   minutes followed by centrifugation to remove the protein A.
Fifty microliters of each sample was run on a 10%
polyacrylamide gel. Results showed that 9 of 10 clones
secreted modified Factor VII.

30   EXAMPLE II
                ANTICOAGULANT ACTIVITY OF MODIFIED FACTOR VII
                The ability of the modified Factor VII protein to
inhibit clotting was measured in a one-stage clotting assay
using wild-type Factor VII as a control. Recombinant proteins
35   were prepared essentially as described above from cells
cultured in media containing 5µg/ml vitamin K. Varying
amounts of the modified Factor VII (from clone 544) or

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recombinant wild-type Factor VII were diluted in 50 mM Tris pH
7.5, 0.1% BSA to 100 μl. The mixtures were incubated with 100
μl of Factor VII-deficient plasma (George King Bio-Medical
Inc., Overland Park, KS) and 200 μl of thromboplastin C (Dade,
5   Miami, FL; contains rabbit brain thromboplastin and 11.8 mM
Ca^{++}). The clotting assay was performed in an automatic
coagulation timer (MLA Electra 800, Medical Laboratory
Automation Inc., Pleasantville, NY), and clotting times were
converted to units of Factor VII activity using a standard
10   curve constructed with 1:5 to 1:640 dilutions of normal pooled
human plasma (assumed to contain one unit per ml Factor VII
activity; prepared by pooling citrated serum from healthy
donors). Using this assay the preparations of modified Factor
VII exhibited no detectable coagulant activity. Table 2 shows
15   results of the assay in terms of clotting times for control
(untransfected) BHK cell-conditioned media (+/- vitamin K),
wild-type Factor VII and two isolates of cells expressing the
modified Factor VII. Factor VII activity is seen as a
reduction in clotting time over control samples.

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Table 2

Sample	Dilution	Clotting Time (sec.)
Control +K	1:5	33.1
	1:10	33.4
Control -K	1:5	34.3
	1:10	33.2
Wild-type Factor VII	1:20	19.0
	1:40	21.5
	1:80	23.3
Modified Factor VII (#6)	1:1	33.5
Modified Factor VII (#10)	1:1	32.5

To determine the effect of the modified Factor VII
45   on plasma factor substrates, preparations of modified Factor

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VII and recombinant wild-type or native Factor VII are
incubated with either Factor X or Factor IX and the activation
thereof monitored by clotting assays or polyacrylamide gel
electrophoresis.

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EXAMPLE III
Ability of Modified Factor VII to Bind Tissue Factor

The ability of the modified Factor VII to compete
with wild-type Factor VII for tissue factor and inhibit its
clotting activity was assessed in a one-step clotting assay in
the presence of a limiting amount of tissue factor
(thromboplastin).

Clotting times were determined in a one-step assay
similar to that described in Example II. A limited amount of
tissue factor, a constant amount of wild type Factor VII, and
increasing amounts of variant Factor VII were used in the
mixing experiments. An inhibition of Factor VII/VIIa
procoagulant activity would be seen as an increase in clotting
time in assays containing increasing amounts of variant Factor
VII.

The amount of Factor VII activity in the test
samples was calculated as a percentage of a standard curve
that measured Factor VII activity in normal pooled plasma.
The standard curve for Factor VII activity was generated using
serial dilutions of normal pooled plasma in phosphate buffered
solution (PBS) that ranged from 1:5 to 1:640. For this
purpose it was assumed that normal plasma contains
approximately 500 ng/ml of Factor VII and this was considered
to be one unit of activity. A mixture of 100 μl Factor VII-
deficient plasma, 100 μl plasma dilution and 200 μl of
thromboplastin-C (Dade, Miami, FL.) was used to measure
clotting time on a MLA Electra 800 automatic timer. To
establish the standard curve, the results were graphed as
percentage of activity (1:5 = 100% activity) versus clotting
time in seconds.

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The assay required that the medium containing the wild type and variant Factor VII be composed of less than one percent serum. The dilutions were made in PBS so that clotting times would fall along the standard curve. A minimum dilution of 1:2 was typical. The final volume was 100 μl. Two different human Factor VII $Ser_{344} \rightarrow$ Ala variants, designated clones "#10" and "#6" were tested in the experiments. The results, set forth in the Table below, show that as the amount of Factor VII variant increased, the percent of Factor VIIa activity decreased.

Table 3: Results of mixing assay with Ser344 → Ala Variants (B4A1 (wild type) medium was used as 100% activity at 10 μl/reaction)

Ser344 → Ala Clone No.	Variant medium amount	B4A1 medium amount	BHK Control*	Percent FVIIa Activity
#10	10 μl	10 μl	0	70
#10	20 μl	10 μl	0	51
#10	30 μl	10 μl	0	43
#10	40 μl	10 μl	0	34
#10	50 μl	10 μl	0	28
#10 (-K)§	20 μl	10 μl	0	78
#6	10 μl	10 μl	0	74
#6	20 μl	10 μl	0	56
#6	30 μl	10 μl	0	46
#6	40 μl	10 μl	0	41
#6	50 μl	10 μl	0	32
#6 (-K)	20 μl	10 μl	0	85
BHK control	0	10 μl	20 μl	91
BHK control (-K)	0	10 μl	20 μl	107

* Untransfected conditioned medium
§ For expression of the Factor VII variant, cells were grown in the presence of vitamin K, except where noted "(-K)".

These experiments showed that variants of Factor VII having a $Ser_{344} \rightarrow$ Ala substitution competed with native Factor VII in a dose dependent fashion and inhibited the procoagulant activity of native Factor VII/VIIa. It can thus be concluded that $Ser_{344} \rightarrow$ Ala variant human Factor VII competes with native human Factor VIIa and consequently inhibits activation of Factor X and/or IX in human plasma.

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EXAMPLE IV
Reaction of Factor VII with PPACK

Recombinant Factor VII was produced in transfected baby hamster kidney cells. The protein was purified and activated as disclosed by Thim et al. (_Biochemistry 27_: 7785-7793, 1988), Brinkous et al. (_Proc. Natl.Acad. Sci. USA 86_: 1382-1386, 1989) and Bjoern and Thim (_Res. Discl. No. 269_, 564, 1986), which are incorporated herein by reference. The cell culture medium was recovered, filtered and diluted to reduce sale concentration. The diluted medium was then fractionated by anion exchange chromatography using an elution buffer containing $CaCl_2$. The Factor VII fraction was recovered and further purified by immunochromatography using a calcium-dependent anti-Factor VII monoclonal antibody. Additional purification was carried out using two anion exchange chromatography steps wherein Factor VII was eluted using $CaCl_2$ and NaCl, respectively. Factor Vlla was recovered in the final eluate.

Recombinant Factor Vlla (1 μM) in 50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$, ph 7.4 was incubated with 20 μM PPack (D-Phenylalanyl-Prolyl-Arginyl Chloromethyl Ketone; Calbiochem, La Jolla, CA) for 5, 20 and 60 minutes. Buffer containing the chromogenic substrate S2288 (H-D-Isoleucine-L-Prolyl-L-Arginine p-nitroanilide; Kabi Vitrum AB, Molndal, Sweden) was then added to obtain a 2.5 fold dilution and a final concentration of 0.3 mM S2288. The generation of p-nitroaniline was measured and compared to results using untreated Factor Vlla as a control. The results indicated that Factor Vlla is fully inactivated after about 60 minutes under these reaction conditions.

EXAMPLE V
Generation of DEGR-Factor VIIa

Recombinant human Factor VIIa was prepared as described in Example IV. Recombinant human Factor VIIa, in 10 mM glycine buffer, pH 8.0, 10 mM $CaCl_2$, 50 mM NaCl, was diluted to a concentration of 1.5 mg/ml. A 10-fold molar

excess of Dansyl-L-Glu-Gly-Arg-Chloromethyl Ketone, DEGRck, (Calbiochem, La Jolla, CA 92037) which had been dissolved with distilled H_2O was added to the Factor VIIa. After a 2 hr incubation at 37°C, a second 10-fold molar excess of DEGRck was added to the mixture and incubated for an additional 2 hr at 37°C. A third 10-fold molar excess of DEGRck was added to the Factor VIIa and incubated for approximately 16 hours at 4°C. The DEGR-Factor VIIa sample was then extensively dialyzed at 4°C against Tris buffered saline (0.05 M Tris-HCl, 0.1 M NaCl, pH 7.5) to remove any free DEGRck.

The final DEGR-Factor VIIa mixture was tested for the presence of free DEGRck in a Factor Xa chromogenic substrate assay. The DEGR-Factor VIIa mixture was added to purified human Factor Xa along with the chromogenic substrate S-2222. This substrate is cleaved specifically by Factor Xa and not by Factor VIIa. Unbound DEGRck in the mixture is able to bind to the Factor Xa and there by inhibit the chromogenic activity of the Factor Xa. Spiking free DEGR-ck into a Factor Xa mixture generated a standard curve to measure the level of free DEGRck in solution versus the inhibition of Factor Xa chromogenic activity. Analysis of the DEGR-Factor VIIa mixture showed that the ratio of free DEGRck:DEGR-Factor VIIa was less than 0.5% following extensive dialysis, thereby ensuring that the inhibition observed by DEGR-Factor VIIa in the various assay systems described below was not due to the presence of free DEGRck.

Example VI
Factor Xa generation on rat smooth muscle cells

Vascular smooth muscle cells were analyzed for the presence of cell-surface tissue factor by measuring the ability of the cells to stimulate the conversion of Factor X to Factor Xa using a chromogenic substrate that is specific for Factor Xa.

Rat vascular smooth muscle cells (Clowes et al., J. Clin. Invest. 93:644-651 (1994)) were plated into 96-well

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culture dishes (American Scientific Products, Chicago, Il.) at 8,000 cells per well in growth media (Table 4).

Table 4

500 ml Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO-BRL,
Gaithersburg, MD.)
10% fetal calf serum (Hyclone, Logan, UT.)
1mM sodium pyruvate (Irvine, Santa Ana, CA.)
0.29 mg/ml L-glutamine (Hazelton, Lenexa,KS.)
1x PSN; (100X is 5 mg/ml penicillin, 5 mg/ml streptomycin,
        10 mg/ml neomycin) (GIBCO-BRL, Gaithersburg, MD.)

After a 48 hour incubation at 37°C the medium was changed to serum free medium (Table 5).

Table 5

250 ml Dulbecco's Modified Eagle's Medium (DMEM)
250 ml Ham's F-12 Medium (Fred Hutchinson Cancer Research
        Center, Seattle, WA)
1mM sodium pyruvate
.29 mg/ml L-glutamine
20 μM transferrin (JRH, Lenexa, KS.)
5 μM insulin (GIBCO-BRL)
16 ng selenium (Aldrich, Milwaukee, WI.)
1 mg/ml bovine serum albumin (Sigma, St. Louis, MO)

The cells were incubated 72 hours at 37°C. After incubation, either PDGF-BB (10 ng/ml) or 10% fetal calf serum was added to the cells to stimulate tissue factor expression (Taubman et al., J. Clin. Invest. 91:547-552, 1993). A parallel set of cells received neither PDGF nor serum to monitor for intrinsic activity of unstimulated cells. After a 6 hour incubation, recombinant human Factor VIIa was added to the cells at a final concentration of 10 nM. One set of cells did not have Factor VIIa added as a negative control. The cells were incubated for 2 hours at 37°C and washed with HEPES buffer (10 mM HEPES, 137 mM NaCl, 4 mM KCl, 5 mM $CaCl_2$, 11 mM glucose, 0.1% BSA). After washing, cells were incubated for 5 min with 50 μl per well of 200 nM plasma-purified human Factor X in a Tris-buffered saline supplemented with 5 mM $CaCl_2$. Twenty-five microliters of 0.5 M EDTA and 25 μl of an 800 μM solution of S-2222 chromogenic substrate (Kabi

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Pharmacia, Franklin, OH) were added to each well. The plates were incubated for 40 min at room temperature, then analyzed at 405 nm using a THERMO MAX Microplate Reader (Molecular Devices, Menlo Park, CA).

Table 6 shows an increase in absorbance for the Factor VIIa treated wells as compared to the control wells (no Factor VIIa added). The increase in absorbance is a direct measurement of the level of Factor Xa generated in the wells and its subsequent cleavage of the chromogenic substrate, releasing the chromophore. The data also demonstrate that the level of chromogenic activity in cells pretreated with either PDGF-BB or 10% fetal calf serum was higher than unstimulated cells.

Table 6

Test Sample	OD_{405}
Control	.043
Intrinsic	.247
PDGF-BB	.360
10% FCS	.342

These results clearly show there is a Factor VIIa-dependent activation of Factor X to Factor Xa on the cell surface of rat vascular smooth muscle cells.

Example VII

Inhibition of cell-surface chromogenic
activity by DEGR-Factor VIIa.

Rat vascular smooth muscle cells were plated into 96-well culture dishes as described above. The cells were cultured for 72 hours in serum free media as described above and treated with the addition of 10% fetal calf serum for 6 hours to stimulate tissue factor expression. After stimulation buffer only (control), 10 nM Factor VIIa, or 10 nM Factor VIIa + 100 nM DEGR-Factor VIIa was added to each well. The cells were incubated for 2 hours at 37°C, then washed with HEPES buffer. After washing, the cells were incubated for 5

minutes with 50 μl per well of 200 nM Factor X in Tris-
buffered saline supplemented with 5 mM $CaCl_2$. Twenty-five
microliters of 0.5 M EDTA and 25 μl of S-2222 (800 μM)
chromogenic substrate (Kabi Pharmacia) were added to each
well. The cells were incubated at room temperature for 40
minutes. Chromogenic activity was analyzed at 405 nm as
described above.

Table 7 shows stimulation of chromogenic activity in
the wells treated with Factor VIIa only, and inhibition of
stimulation when DEGR-Factor VIIa was coincubated with the
Factor VIIa. These results demonstrate that DEGR-Factor VIIa
acts as a competitive antagonist for Factor VIIa binding,
thereby inhibiting the activation of Factor X to Factor Xa and
the subsequent cleavage of the S-2222 chromogen.

Table 7

Test Sample	OD_{405}
Control	.035
Factor VIIa	.342
Factor VIIa + DEGR-Factor VIIa	.073

Example VIII
Dose dependent inhibition by DEGR-Factor VIIa of cell
surface chromogenic activity on rat smooth muscle cells.

Rat vascular smooth muscle cells were plated into
96-well culture dishes at 4,000 cells per well in growth
medium supplemented with 1% fetal calf serum (as in Table 4
without 10% fetal calf serum). After 5 days the medium was
removed, and either increasing concentrations of Factor VIIa
alone or 10 nM Factor VIIa with increasing concentrations of
DEGR-Factor VIIa were added to the cells. The cells were
incubated with the Factor VII mixtures for 2 hours at 37°C.
After incubation, the cells were washed and incubated with 50
μl of 200 nM Factor X in tris buffered saline for 5 minutes at
room temperature. Each well had 25 μl of 0.5 M EDTA and 25 μl

of 800 μM S-2222 (Kabi Pharmacia) added to it, and the plates were incubated for 40 minutes at room temperature. Chromogenic activity was analyzed at 405 nm in a microplate reader as described above.

Table 8 shows a dose-dependent increase in chromogenic activity with increasing amounts of Factor VIIa added to the wells. When the mixture of DEGR-Factor VIIa with 100 nM Factor VIIa was added to the cells (Table 9) there was a dose dependent inhibition in chromogenic activity. A 1:1 molar ratio of DEGR-Factor VIIa:Factor VIIa inhibited approximately 95% of the chromogenic activity. These data suggest that DEGR-Factor VIIa has a significantly higher affinity for cell-surface tissue factor than native Factor VIIa. If DEGR-Factor VIIa and Factor VIIa had equal affinity for binding tissue factor then the level of inhibition observed when the two molecules were added to the cells in an equal molar ratio would not have been as high.

Table 8

Factor VIIa Conc. (nM)	OD_{405}
.10	.005
.39	.025
1.56	.058
6.25	.111
25.00	.154
100.00	.208

Table 9 shows the dose dependent inhibition of Factor Xa chromogenic activity on rat smooth muscle cells by DEGR-Factor VIIa. Increasing concentrations of DEGR-Factor VIIa were coincubated with 100 nM Factor VIIa, and the Factor Xa chromogenic activity determined using chromogenic substrate S-2222.

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Table 9

DEGR-Factor VIIa Conc. (nM)	OD_{405}
.00	.208
.39	.176
1.56	.116
6.25	.073
25.00	.026
100.00	.014

Example IX

Inhibition of Factor Xa generation by
DEGR-Factor VIIa in a soluble tissue factor assay.

The conversion of Factor X to Factor Xa using
purified recombinant soluble tissue factor was established
using a chromogenic assay. Tissue factor was expressed and
purified from Saccharomyces cerevisiae (Shigematsu et al., J.
Biol. Chem. 267:21329-21337, 1992). Soluble tissue factor was
purified and characterized by Dr. Walt Kisiel (University of
New Mexico, Albequerque, NM). A reaction mixture containing
65.9 μl of soluble tissue factor (2.2 μM), 29.0 μl of PCPS (1
mM, Sigma, St. Louis, MO), 29.5 μl human Factor X (4.1 μM),
2.77 ml Hank's buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 2.7 mM
KCl, 5 mM $CaCl_2$, 0.1% BSA) was prepared. Forty microliter of
tissue factor/Factor X mixture, 25 μl Factor VIIa diluted with
TBS and 25 μl of DEGR-Factor VIIa diluted with TBS were added
to each well of a 96-well microtiter plate. A control using
40 1 of tissue factor/Factor X mixture; 25 μl Factor VIIa
diluted with TBS, and 25 μl of TBS only was included. Ten
microliters of S-2222 (4 mM) chromogenic substrate was added
to the reaction mixture in the wells and incubated at room
temperature for 2-10 minutes. Results were analyzed at 405 nm
in a microplate reader as described above.

Determination of a standard curve for Factor VIIa
activation of Factor X was made using increasing
concentrations of Factor VIIa added in the absence of DEGR-
Factor VIIa. The results, presented in Table 10, show that

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there is a dose-dependent increase in chromogenic activity with increasing amounts of Factor VIIa added to the reaction mixture. The simultaneous addition of varying amounts of DEGR-Factor VIIa and 100 nM Factor VIIa led to a dose dependent decrease in chromogenic activity (Table 11). These data demonstrate that DEGR-Factor VIIa acts as a competitive antagonist for native Factor VIIa binding to soluble tissue factor, and thereby inhibits the generation of Factor Xa as measured by the decrease in chromogenic activity towards the chromogenic substrate S-2222.

Table 10

Stimulation of Factor Xa chromogenic activity with increasing concentrations of Factor VIIa added to soluble tissue factor. Changes in optical density were measured using chromogenic substrate S-2222.

Factor VIIa Conc (nM)	OD_{405}
.78	.168
1.56	.288
3.12	.478
6.25	.694
12.50	.764
25.00	.790
50.00	.738
100.00	.770

Table 11

Inhibition of Factor Xa chromogenic activity by the addition of DEGR-Factor VIIa to soluble tissue factor in the presence of native Factor VIIa is measured. Changes in optical density were measured using the chromogenic substrate S-2222.

DEGR-Factor VIIa Conc. (nM)	OD_{405}
0.	810
50.	750
100.	609
200.	296
400.	167
800.	083
1600.	055

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Example X

Inhibition of coagulation by DEGR-Factor VIIa.

Standard clotting assays to monitor the effect of DEGR-
Factor VIIa on clotting time were prepared as follows: 100 μl
of normal baboon plasma, collected with sodium citrate as
anticoagulant, was added to 100 μl of varying concentrations
of DEGR-Factor VIIa diluted in TBS (20 mM Tris, pH 7.4, 150 mM
NaCl). The samples were mixed and briefly incubated at 37°C.
The samples were added to an Electra 800 Automatic Coagulation
Timer (Medical Laboratories Automation, Pleasantville, NY).
After incubation, 200 μl of a tissue factor preparation
containing 25 mM $CaCl_2$ was added to the DEGR-Factor VIIa
preparations. A tissue factor preparation was made as a
saline extract of baboon brain that from freshly frozen brain
tissue and characterized for its ability to initiate
coagulation in baboon plasma. A concentration of tissue
factor that gave a clotting time of about 40 seconds was
selected.

The data, presented in Table 12, demonstrates a
dose-dependent increase in clotting time due to the addition
of DEGR-Factor VIIa. A dose as low as 1 μg/ml of DEGR-Factor
VIIa in plasma resulted in a significant increase in clotting
time.

Table 12

Dose dependent increase in clotting time due to DEGR-
Factor VIIa.

DEGR-Factor VIIa (μg/ml plasma)	Clotting time (seconds)
0	40.7
0.5	46.2
1.0	50.8
2.5	64.5
5.0	108.1
10.0	158.4

Example XI
Inhibition of platelet accumulation
with DEGR-Factor VIIa.

DEGR-Factor VIIa was analyzed for its ability to inhibit platelet accumulation at sites of arterial thrombosis due to mechanical injury in non-human primates. A model of aortic endarterectomy was utilized in baboons, essentially as described by Lumsden et al. (Blood 81:1762-1770, 1993). A section of baboon aorta 1-2 cm in length was removed, inverted and scraped to remove the intima of the artery and approximately 50% of the media. The artery was reverted back to its correct orientation, cannulated on both ends and placed into an extracoporeal shunt in a baboon, thereby exposing the mechanically injured artery to baboon blood via the shunt. Just prior to opening of the shunt to the circulating blood, ^{111}In-labeled autologous platelets were injected intravenously into the animal. The level of platelet accumulation at the site of the injured artery was determined by real-time gamma camera imaging.

Evaluation of DEGR-Factor VIIa for inhibition of platelet accumulation was done using bolus injections of DEGR-Factor VIIa or saline control and were given just prior to the opening of the shunt. The injured arteries were measured continuously for 60 minutes. A dose of 0.005 mg/kg of DEGR-Factor VIIa inhibited platelet accumulation. At a 1.0 mg/kg bolus injection, approximately 90% of platelet accumulation was inhibited at 1 hour post drug administration.

These data show that inhibition of tissue factor with DEGR-Factor VIIa can significantly inhibit the development of platelet-rich thrombi in a nonhuman primate model of acute vascular injury.

It is evident from the foregoing that compositions of Factor VII or VII having modified catalytic sites are provided which are able to bind tissue factor yet are substantially unable to activate Factors X and IX. As modified Factor VII acts specifically to interrupt the

clotting cascade without degrading or consuming clotting
factors, it can be expected that administration of modified
Factor VII preparations will be accompanied by fewer
undesirable side effects than experienced with current
5   therapies. Further, the modified Factor VII described herein
may readily be produced by recombinant means. Thus efficacy,
convenience and economics of lower dosages and less frequent
administration, and a relative lack of toxicity are among the
advantages conferred by the compositions of the present
10   invention.

        Although the foregoing invention has been described
in some detail by way of illustration and example for purposes
15   of clarity of understanding, it will be obvious that certain
changes and modifications may be practiced within the scope of
the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: ZymoGenetics, Inc. et al.
        (B) STREET: 4225 Roosevelt Way N.E.
        (C) CITY: Seattle
        (D) STATE: Washington
        (E) COUNTRY: United States of America
        (F) POSTAL CODE (ZIP): 98105
        (G) TELEPHONE: (206)547-8080
        (H) TELEFAX:
        (I) TELEX:

    (ii) TITLE OF INVENTION: Modified Factor VII

    (iii) NUMBER OF SEQUENCES: 4

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (v) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE: 23-MAY-1994
        (C) CLASSIFICATION:

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 08/065,725
        (B) FILING DATE: 21-MAY-1993

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 07/662,920
        (B) FILING DATE: 28-FEB-1991

    (vii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Parmelee, Steven W.
        (B) REGISTRATION NUMBER: 31,990
        (C) REFERENCE/DOCKET NUMBER: 13952-8-1PC

    (viii) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 206-467-9600
        (B) TELEFAX: 415-543-5043

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 2422 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: N

    (iv) ANTI-SENSE: N

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 28..1420
        (D) OTHER INFORMATION: /codon_start= 28
                        /product= "Factor VII"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GTC TTC GTA ACC CAG GAG GAA GCC CAC GGC GTC CTG CAC CGG CGC CGG     151
Val Phe Val Thr Gln Glu Glu Ala His Gly Val Leu His Arg Arg Arg
        -15             -10                 -5

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Asp Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp
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Gln Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg
    65                  70                  75

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80                  85                  90                      95

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Cys Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys
                115                 120                 125

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            130                 135                 140

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                180                 185                 190

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Ser Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His
                340             345             350

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            355             360             365

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ATAAACGGCT GCGTCTCCTC CGCACACCTG TGGTGCCTGC CACCCAAAAA AAAAAAAAAA 2416

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(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 444 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein.

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

   (iii) HYPOTHETICAL: N

    (iv) ANTI-SENSE: N

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 15 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: N

    (iv) ANTI-SENSE: N

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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WHAT IS CLAIMED IS:

       1.   A method for inhibiting tissue factor activity in a patient comprising administering to the patient a therapeutically effective dose of a composition comprising Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.

       2.   A method according to claim 1, wherein the modification comprises reaction of the Factor VII with a serine protease inhibitor.

       3.   A method according to claim 2, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.

       4.   A method according to claim 3, wherein the protease inhibitor is a peptide halomethyl ketone selected from D-Phe-Pro-Arg chloromethyl ketone or Dansyl-Glu-Gly-Arg chloromethyl ketone.

       5.   A method according to claim 1, wherein the Factor VII modification comprises at least one amino acid substitution, insertion, or deletion in a catalytic triad of Ser, Asp, and His.

       6.   A method for inhibiting vascular restenosis in a patient comprising administering to the patient a therapeutically effective dose of a composition comprising Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.

       7.   A method according to claim 6, wherein the modification comprises reaction of the Factor VII with a serine protease inhibitor.

8. A method according to claim 7, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.

9. A method according to claim 8, wherein the protease inhibitor is a peptide halomethyl ketone selected from D-Phe-Pro-Arg chloromethyl ketone or Dansyl-Glu-Gly-Arg chloromethyl ketone.

10. A method for inhibiting platelet deposition in a patient comprising administering to the patient a therapeutically effective dose of a composition comprising Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX.

11. A method according to claim 10, wherein the modification comprises reaction of the Factor VII with a serine protease inhibitor.

12. A method according to claim 10, wherein the protease inhibitor is an organophosphor compound, a sulfanyl fluoride, a peptide halomethyl ketone, or an azapeptide.

13. A method according to claim 12, wherein the protease inhibitor is a peptide halomethyl ketone selected from D-Phe-Pro-Arg chloromethyl ketone or Dansyl-Glu-Gly-Arg chloromethyl ketone.

14. A method according to claim 10, wherein the Factor VII modification comprises at least one amino acid substitution, insertion, or deletion in a catalytic triad of Ser, Asp, and His.

15. Use of a composition of a pharmacologically acceptable Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma

Factor X or IX, for the treatment of inhibiting vascular restenosis in an individual.

16. Use of a composition of a pharmacologically acceptable Factor VII having at least one modification in its catalytic center, which modification substantially inhibits the ability of the modified Factor VII to activate plasma Factor X or IX, for inhibiting platelet deposition in an individual.
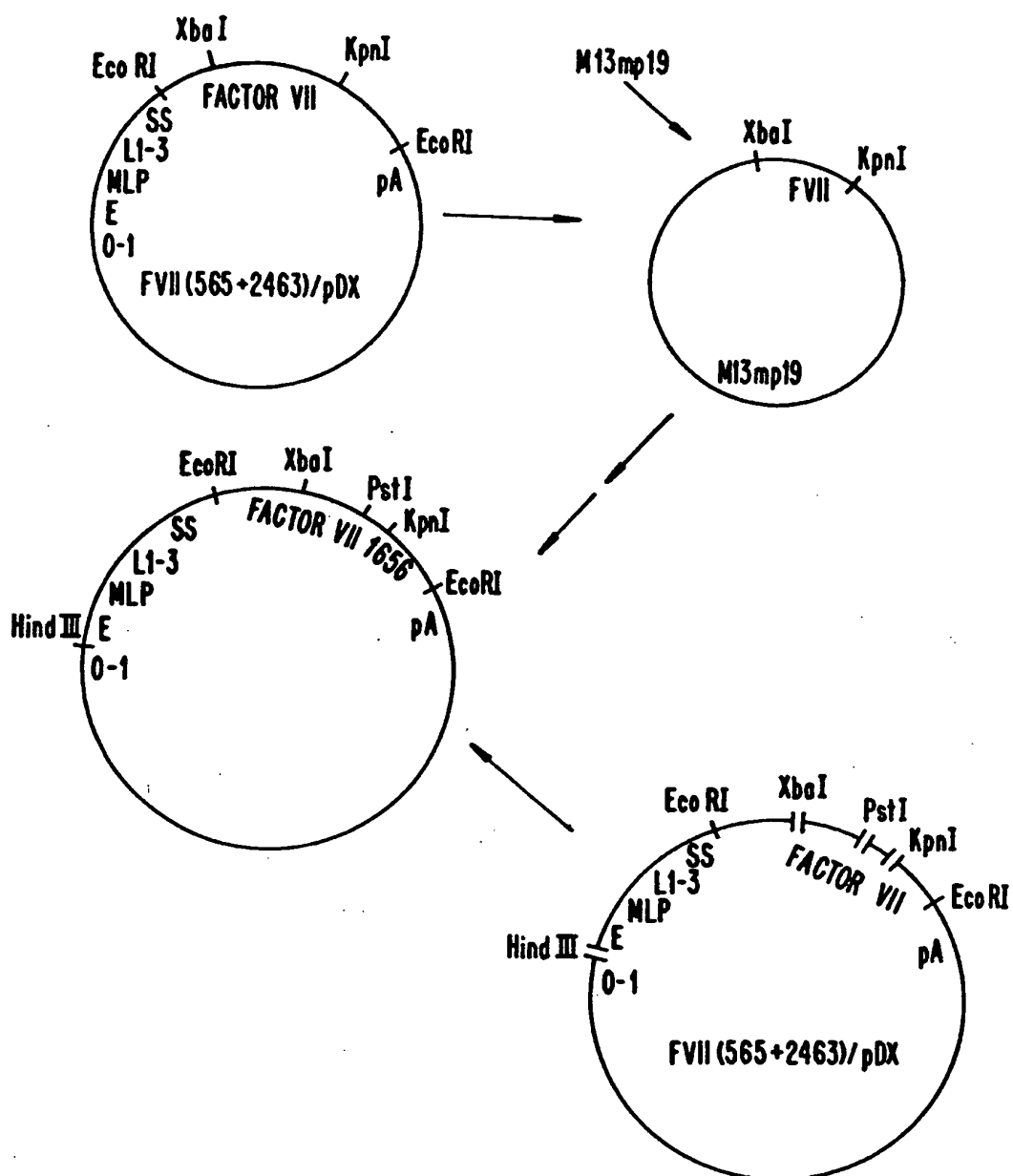
FIGURE

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   A61K37/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 15686 (ZYMOGENETICS) 17 September 1992 * see the whole document *	1-5
A	* " *	6-15
A	WO,A,91 11514 (ZYMOGENETICS) 8 August 1991 * see claims 1-23 *	1-15

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 October 1994	27 -10- 1994

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Isert, B

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat l Application No
PCT/US 94/05779

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9215686	17-09-92	AU-A-	1449892	06-10-92
		EP-A-	0575464	29-12-93
		JP-T-	6504678	02-06-94
WO-A-9111514	08-08-91	AU-B-	651573	28-07-94
		AU-A-	7300291	21-08-91
		EP-A-	0521873	13-01-93
		JP-T-	5500161	21-01-93
		US-A-	5288629	22-02-94